

Mono(2-Ethyl-5-Hydroxyhexyl) Phthalate and Mono-(2-Ethyl-5-Oxohexyl) Phthalate as Biomarkers for Human Exposure Assessment to Di-(2-Ethylhexyl) Phthalate

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Exposure to di-(2-ethylhexyl) phthalate (DEHP) is prevalent based on the measurement of its hydrolytic metabolite mono-(2-ethylhexyl) phthalate (MEHP) in the urine of 78% of the general U.S. population studied in the 1999–2000 National Health and Nutrition Examination Survey (NHANES). However, despite the high level of production and use of DEHP, the urinary MEHP levels in the NHANES samples were lower than the monoester metabolites of phthalates less commonly used than DEHP, suggesting metabolic differences between phthalates. We measured MEHP and two oxidative DEHP metabolites, mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) to verify whether these other metabolites account for a greater proportion of DEHP metabolic products in 127 paired human urine and serum samples. We found that the urinary levels of MEHHP and MEOHP were 10-fold higher than levels of MEHP; concentrations of urinary MEOHP and MEHHP were strongly correlated ($r = 0.928$). We also found that the serum levels of MEOHP and MEHHP were comparatively lower than those in urine. Furthermore, the glucuronide-bound conjugates of the oxidative metabolites were the predominant form in both urine and serum. MEOHP and MEHHP cannot be formed by serum enzymes from the hydrolysis of any contamination from DEHP potentially introduced during blood collection and storage. Therefore, concentrations of MEHHP and MEOHP in serum may be a more selective measure of DEHP exposure than is MEHP. However, additional data on the absorption, distribution, metabolism, and elimination of these oxidative metabolites are needed to completely understand the extent of DEHP exposure from the serum concentrations of oxidative DEHP metabolites. **Key words:** DEHP, MEHHP, MEHP, MEOHP, phthalate metabolites, phthalates. *Environ Health Perspect* 112:327–330 (2004). doi:10.1289/ehp.6663 available via <http://dx.doi.org/> [Online 18 November 2003]

Di-(2-ethylhexyl) phthalate (DEHP), one of the most widely used phthalates, is a primary component in polyvinyl chloride plastics used in numerous household products, toys, floor tiles, furniture upholstery, blood storage bags, and medical devices, among other products [Agency for Toxic Substances and Disease Registry (ATSDR) 2002; Faouzi et al. 1999]. Therefore, the potential for human exposure to DEHP is high. DEHP is not chemically bound in the plastics; hence, it can be leached to the environment during the manufacturing process and product use and after disposal (ATSDR 2002; Sharman et al. 1994). The general population is exposed to DEHP in food, water, and air through inhalation and ingestion (ATSDR 2002; Meek and Chan 1994; Sharman et al. 1994). Medical patients receiving transfusions (Peck et al. 1979) or dialysis (Faouzi et al. 1999) or those undergoing apheresis may be more exposed to DEHP than the general population (Ono et al. 1975). DEHP is not classifiable as to its carcinogenicity to humans by the International Agency for Research on Cancer (IARC 2000), but it has been designated a carcinogen in experimental animals (IARC 2000; Kluwe et al. 1982). Moreover, results from animal toxicologic studies have demonstrated

endocrine-modulating effects from high doses of DEHP (Gray et al. 1999). DEHP alters thyroid structure and activity (Gray et al. 1999; Hinton et al. 1986) in male Wistar rats and produces reproductive and developmental toxicities in rodents (Kavlock et al. 2002). In male rodents, the testes are a primary target tissue, and exposure to high doses of DEHP results in decreased testicular weights and tubular atrophy (ATSDR 2002).

Metabolism of DEHP involves hydrolysis to mono(2-ethylhexyl) phthalate (MEHP) and formation of a glucuronide MEHP conjugate. The glucuronide conjugate and the free monoester can be excreted in urine and feces or, alternatively, can undergo ω or (ω -1) hydroxylation or β elimination to form several oxidative metabolites (Albro et al. 1973; ATSDR 2002). These oxidative metabolites can also be excreted in urine and feces as the free monoester or glucuronide conjugates.

Several methods of measuring DEHP in biologic matrices have been published (Albro et al. 1984; Luster et al. 1978). However, because DEHP is a ubiquitous laboratory contaminant, field blanks show concentrations similar to those in the matrices under study, thus limiting the accuracy of the measurements. Measuring metabolites instead of the

parent phthalate overcomes this limitation and makes accurate exposure assessment possible (Blount et al. 2000a; Kato et al. 2003; Silva et al. 2003a). We have used urinary MEHP measurements in our previous studies for exposure assessment of DEHP [Blount et al. 2000b; Brock et al. 2002; Centers for Disease Control and Prevention (CDC) 2003]. However, the concentrations of MEHP we found in human urine were lower than those of other phthalate monoesters, particularly the phthalates with fewer carbons in the alkyl side chains, despite the likely higher availability of DEHP. This could result from several factors, including differences in exposure, systemic absorption from the gastrointestinal tract (White et al. 1980), metabolism (Albro and Moore 1974; Williams and Blanchfield 1974, 1975), and excretion. Phthalates with short alkyl side chains undergo oxidative metabolism to a lesser extent (Albro and Moore 1974; Williams and Blanchfield 1974, 1975), compared with phthalates with long alkyl side chains. The differences in metabolism among phthalates could result in urinary excretion of comparatively higher levels of the monoester metabolites of the phthalates with short alkyl chains than for DEHP, which is initially metabolized to MEHP and then further to several oxidative metabolites (Albro et al. 1973; ATSDR 2002).

Although concentrations in urine samples provide invaluable exposure information, the concentrations of excreted substances are dependent on water intake. Therefore, toxicokinetics are easier to interpret from measurements in blood (e.g., serum, plasma), and serum markers of DEHP exposure might prove more useful. However, we recently have shown that blood measurements of phthalate monoesters are susceptible to contamination from the parent phthalates, which are hydrolyzed to their respective monoesters

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by serum enzymes (Kato et al. 2003). If the contamination is not properly eliminated, the measured concentrations in serum of the phthalate monoesters, but not of the oxidative metabolites, will be artificially elevated.

To address these problems, we evaluated MEHP and two oxidative metabolites, mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), as markers for exposure to DEHP in 127 paired human urine and serum samples.

Materials and Methods

MEHHP, MEOHP, MEHP (Figure 1), DEHP, ¹³C₄-monobutyl phthalate (¹³C₄-mBP), ¹³C₄-MEHP, and ¹³C₄-4-methylumbelliferone (¹³C₄-MeUmb) were purchased from Cambridge Isotopes Laboratories Inc. (Andover, MA, USA). Acetonitrile and water (HPLC grade) were purchased from Tedia (Fairfield, OH, USA), MeUmb and its glucuronide (MeUmb-glu) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). β-Glucuronidase (*Escherichia coli*-K12) was purchased from Roche Biomedical (Mannheim, Germany). Stock solutions of standards (MEHP, MEOHP, MEHHP, and MeUmb) and internal standards (¹³C₄-mBP, ¹³C₄-MEHP, and ¹³C₄-MeUmb) were prepared in acetonitrile (Blount et al. 2000a). The internal standard spiking solution consisted of ¹³C₄-mBP (20 ng) and ¹³C₄-MEHP (12 ng). ¹³C₄-mBP was used as the internal standard

for MEOHP and MEHHP because of their similar retention on the HPLC column. ¹³C₄-MEHP was used as the internal standard for MEHP.

High (~200 ng/mL) and low (~25 ng/mL) quality control (QC) pools were prepared from split pooled human urine and bovine serum. The urine and serum were spiked with the desired amounts of MEOHP, MEHHP, MEHP, and phosphoric acid (only the serum pools), mixed well, and divided into aliquots. A reagent blank and one each of the concentrations of QC materials were analyzed during each analytical run to ensure proper operation of the method and the validity of the resulting data.

To determine the effect of serum lipases on MEHP, MEHHP, and MEOHP levels, DEHP-spiked (2,000 ng/mL) serum aliquots (1 mL) were incubated at 37°C. We then measured the concentrations of MEHP, MEHHP, and MEOHP in the DEHP-spiked serum after adding phosphoric acid (Kato et al. 2003) at different time intervals to evaluate the effect of the acid on the enzyme activity.

The analytical methods for measuring phthalate metabolites in urine and serum were adapted from our previously developed methods and modified to meet our requirements for the analysis of MEOHP and MEHHP (Blount et al. 2000a; Kato et al. 2003; Silva et al. 2003a). For the free metabolite (nonglucuronidated) analysis, the β-glucuronidase

enzyme treatment for both unknown samples and QC materials was eliminated. The urine and serum samples (1 mL) were spiked with ¹³C₄-labeled internal standards and MeUmb-glu (to evaluate the completion of the hydrolysis), and the phthalate metabolites were extracted from the matrix by solid-phase extraction. The final eluate was concentrated and resuspended in water. The analytes were chromatographically separated (HP 1100, Agilent Technologies, Wilmington, DE, USA) on a Keystone phenyl Betasil column (Keystone Scientific, Bellefonte, PA, USA) using a nonlinear water:acetonitrile gradient and analyzed by tandem mass spectrometry on an API 3000 (Applied Biosystems, Foster City, CA, USA) using electrospray ionization. The limits of detection (LODs) were calculated as 3S₀, where S₀ is the standard deviation value as the concentration approaches zero (Taylor 1987). S₀ was determined from the replicate analysis of low-level standards. The relative standard deviations ranged from 8% to 13%. The LODs in urine were 1.2 ng/mL (MEOHP), 1.6 ng/mL (MEHHP), and 0.9 ng/mL (MEHP) (Silva et al. 2003a); the LODs in serum were 1.3 ng/mL (MEHP) (Kato et al. 2003), 1.4 ng/mL (MEOHP), and 1.9 ng/mL (MEHHP).

All of the samples, blanks, standards, and QC materials were processed identically using

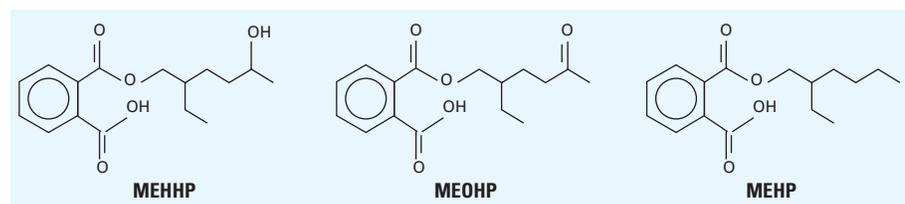


Figure 1. DEHP metabolites used as markers of human exposure to DEHP.

Table 1. The urinary and serum levels (ng/mL) of MEHP, MEOHP, and MEHHP in a group of 127 persons.

Phthalate monoester	Matrix	Percentage detects	GM ^a	Selected percentiles				
				5th	25th	50th	75th	95th
MEHP								
Total	Urine	35	ND	< LOD	< LOD	< LOD	2.3	20.4
	Serum ^b	88	3.9	< LOD	2.8	4.0	5.9	15.2
Free	Urine	1	ND	< LOD	< LOD	< LOD	< LOD	< LOD
	Serum ^b	84	2.5	< LOD	2.2	2.8	3.4	5.0
MEOHP								
Total	Urine	91	14.8	< LOD	5.1	15.6	40.1	243
	Serum	18	ND	< LOD	< LOD	< LOD	< LOD	3.4
Free	Urine	62	5.6	< LOD	1.3	3.9	9.6	48.8
	Serum	3	ND	< LOD	< LOD	< LOD	< LOD	< LOD
MEHHP								
Total	Urine	95	19.3	< LOD	8.8	17.4	41.1	220
	Serum	44	ND	< LOD	< LOD	< LOD	< LOD	3.1
Free	Urine	38	ND	< LOD	< LOD	< LOD	< LOD	5.8
	Serum	4	ND	< LOD	< LOD	< LOD	< LOD	< LOD

Abbreviations: GM, geometric mean; ND, not determined (geometric means were determined only when the frequency of detection was ≥ 60%).

^aLOD/√2 was used if the concentration was < LOD. ^bConcentrations may be artificially overestimated because of serum lipase activity.

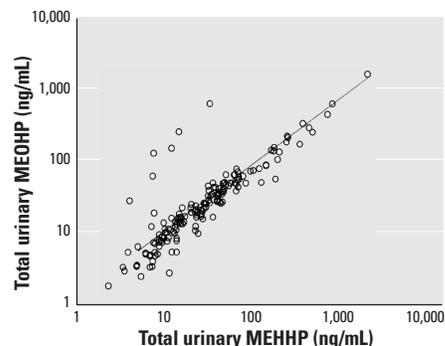


Figure 2. Pearson correlation analyses between the concentrations of urinary MEHHP and MEOHP ($r = 0.928, p < 0.0001$).

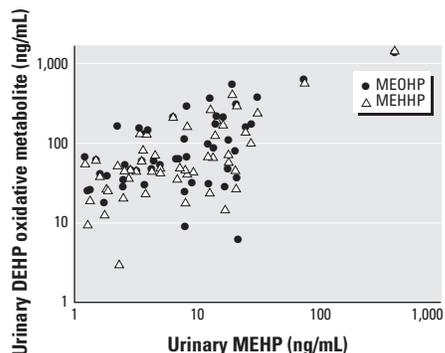


Figure 3. Correlation between urinary MEHP and MEHHP ($r = 0.892$) and urinary MEHP and MEOHP ($r = 0.878$).

the Analyst software (Applied Biosystems) of the API 3000. Each ion of interest in the chromatogram was automatically selected and integrated. The peak integrations were checked for errors and corrected manually, if necessary. A calibration curve of peak area ratio of analyte to ^{13}C -labeled internal standard peak area ratio (after compensating for isotopic impurity of labeled internal standard) versus the reciprocal of concentration ($1/x$) was used for quantification. Samples with values below the LODs were assigned a concentration equal to the LOD divided by the square root of 2 for the statistical analyses. The measured values were used in the figures. Statistical significance was set at $p < 0.05$. Statistical analysis of the data was performed using SAS software (SAS Institute, Cary, NC, USA).

Subjects. The samples analyzed for this study were collected from 176 U.S. residents during 2001 from a demographically diverse population group with no documented exposure to DEHP; 127 were paired urine and serum samples. The samples were collected at different times throughout the day, and the urine samples were not necessarily

first-morning voids. Serum and urine samples were collected on the same day. Informed consent was obtained from all participants.

Results and Discussion

The concentrations of the three DEHP metabolites MEHP, MEOHP, and MEHHP varied widely among the samples tested. Concentrations of MEOHP and MEHHP in urine appeared approximately 10-fold higher than the concentrations of MEHP (Table 1). In addition, the urinary concentrations of MEOHP and MEHHP were highly correlated (Figure 2; $r = 0.928$, $p < 0.0001$), in agreement with our previous findings (Barr et al. 2003). We also observed a good correlation between urinary MEHP and MEHHP levels ($r = 0.892$, $p < 0.0001$) and between MEHP and MEOHP levels ($r = 0.878$, $p < 0.0001$) (Figure 3). The correlations did not improve significantly after adjusting for creatinine to correct for urine dilution (data not shown).

The oxidative metabolites may be glucuronidated in the body to increase their water solubility, hence facilitating the urinary excretion. Furthermore, glucuronidation is a major

mechanism for elimination of the potentially toxic MEHP (Silva et al. 2003b). In this study, 81% of the participants excreted $> 95\%$ urinary MEHHP in its glucuronidated form, whereas 52% of the participants excreted $> 95\%$ urinary MEOHP as the glucuronide (Figure 4). These data suggest that MEOHP and MEHHP are excreted in urine mainly in their conjugated form. We also found a good correlation between the free and total urinary levels of the oxidative metabolites (Figure 5). The fact that the levels of these metabolites in their free form did not plateau suggests that the saturation or inhibition of the enzyme catalyzing the glucuronidation reaction did not occur at the exposed concentrations.

We also quantified the levels of these same DEHP metabolites in serum (Table 1). The oxidative metabolites were detected in $< 50\%$ of serum specimens. For specimens with detectable concentrations of MEOHP and MEHHP, the serum concentrations were lower than the urinary concentrations (Table 1). The lower levels in serum compared with those in urine for nonpersistent compounds, including the phthalate metabolites, are common (Barr et al. 1999, 2002; Silva et al. 2003b). Interestingly, the percentage of samples with detectable levels of free MEOHP and MEHHP in serum was much lower than for total MEOHP and MEHHP, suggesting serum MEOHP and MEHHP were found predominantly in their glucuronidated forms.

We previously reported (Kato et al. 2003) that a treatment of the serum samples with acid was required at sample collection to denature serum enzymes that convert the phthalate diesters (also present in the sample as a result of contamination) to their respective hydrolytic monoester metabolites. The serum samples analyzed for this study were archived samples. We made no attempt to eliminate the lipase activity that might have falsely elevated the serum MEHP levels from hydrolysis of DEHP by serum lipases (Kato et al. 2003) if

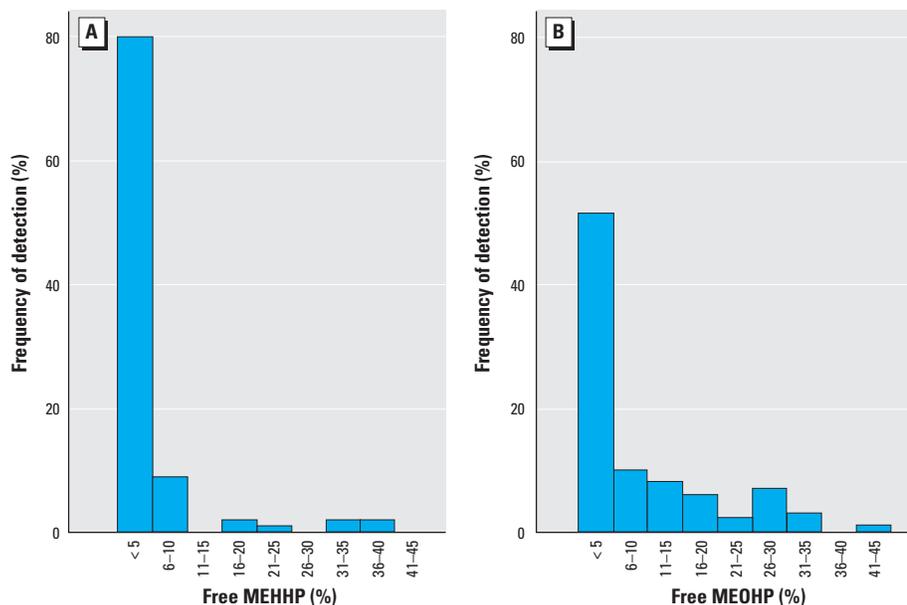


Figure 4. Frequency of detection of free urinary MEHP and MEOHP.

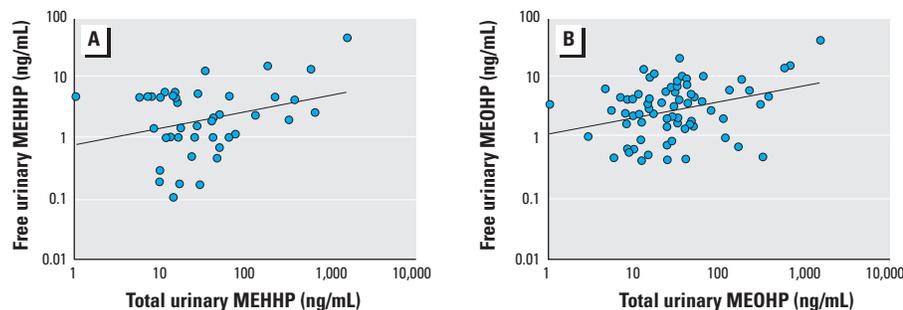


Figure 5. Pearson correlation analyses (A) between the concentrations of total and free urinary MEHP ($r = 0.792$, $p < 0.0001$) and (B) between total and free urinary MEOHP ($r = 0.821$, $p < 0.0001$).

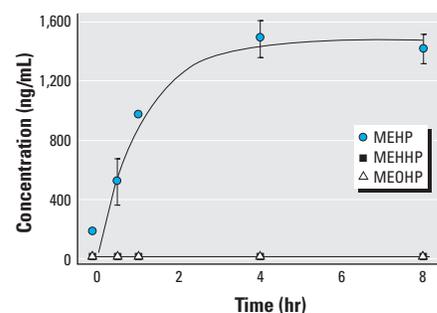


Figure 6. Concentrations of MEHP, MEHHP, and MEOHP in serum at different time intervals after spiking with 2,000 ng/mL DEHP. The error bars represent the SD of triplicate measurements. The concentration of MEHP in DEHP-spiked serum samples increased with time, whereas the concentrations of MEOHP and MEHHP remained unchanged.

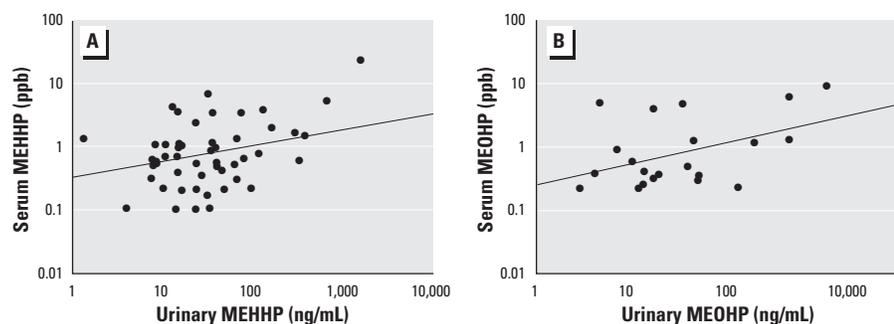


Figure 7. Pearson correlation analyses between the concentrations of (A) total urinary MEHHP and total serum MEHHP ($r = 0.796$, $p < 0.0001$) and (B) total urinary MEOHP and total serum MEOHP ($r = 0.615$, $p < 0.0001$).

preanalytical DEHP contamination of the samples had occurred. Therefore, the levels of MEHP reported in the present study include an unknown contribution from the monoester formed from the lipase-induced hydrolysis of possible contaminant DEHP incorporated in the specimen during the sampling process (Table 1). Because the oxidative phthalate metabolites cannot be formed from the enzymatic cleavage of DEHP, their measurements in serum are not affected by DEHP contamination (Figure 6). Consequently, serum measurements of MEOHP and MEHHP would not be overestimated, even in the presence of high levels of contaminant DEHP. However, to maximize the utility of serum MEOHP and MEHHP as markers of exposure to low levels of DEHP, improvement of the sensitivity of the serum analytical measurements would be necessary.

We observed an association between the levels of MEHHP and MEOHP in urine and serum ($p < 0.001$). The serum MEHHP and MEOHP levels tended to increase with increasing urinary concentrations (Figure 7). Unlike in urine, in serum MEHP was found more often than MEHHP or MEOHP. Because the lipase activity in serum was not eliminated, the MEHP measurements should be interpreted with caution. Additional studies where the serum lipase activity is eliminated should be conducted to determine if this finding is an artifact of contamination.

In summary, our data suggest that MEHHP and MEOHP are excreted in the urine predominantly as glucuronide conjugates and at higher concentrations than MEHP. The higher frequency of detection of MEOHP and MEHHP than of MEHP in urine and the fact that the urinary levels of MEOHP and MEHHP were approximately 10-fold higher than MEHP suggest that MEOHP and

MEHHP may be more sensitive urinary markers of DEHP exposure and may allow low-level exposures to be more readily detected. However, because MEHP is believed to be responsible for the biologic activity attributed to DEHP exposure, MEHP measurements may be more relevant in studies investigating associations between DEHP exposure and adverse health outcomes. Because no information about the biologic activity of the oxidative metabolites is available, further research to establish the bioactivity of MEHHP and MEOHP is warranted.

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